IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Min-Bo Wang et al.	Group Art Unit: 1635
Application No.: 09/287,632	Examiner: JANE J ZARA
Filed: April 7, 1999	Confirmation No.: 6526
For: METHODS AND MEANS FOR OBTAINING MODIFIED PHENOTYPES	

DECLARATION BY DR. MICHAEL METZLAFF UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Michael Metzlaff, hereby states as follows:

1. I am a citizen of Germany.

2. I have received a PhD degree from the University of Halle (Germany) in 1983. I am an expert in the field of the plant molecular biology, particularly the field of post-transcriptional gene silencing. I have authored and co-authored several scientific publications in this field including Metlaff et al. 1997 Cell, Vol. 88 pages 845-854, 1997. My curriculum vitae including a list of publications is attached as ANNEX 1.

3. I am currently employed by BAYER BIOSCIENCE N.V., which is a licensee of the US patent application 09/287,632 (referred below as "the Application").

4. I have read the Application.

5. I have also read the USPTO Official Action, dated September 19, 2008 and I understand that the examined claims have been rejected as allegedly being obvious over the combined disclosures of Metzlaff et al. (Cell, Vol. 88 pages 845-854, 1997) or Flavell et al. (Proc. Natl. Acad. Sci., Vol 91, pages 3490-3496, 1994) or Stam et al. (Annals of Botany, Vol 79., pages 3-12, 1997) in combination with Brown et al. (US patent 5,859,347) or Lusky et al. (US Patent 6,350,575).

6. I understand that the Brown et al. or Lusky et al. references are not recited in connection with the state of the art in the gene-silencing field and are only relied upon to demonstrate that in certain circumstances introns had been used in expression constructs in the prior art, and I will not further comment on these publications, except to say, that I find no reason in these references that would have led a person of ordinary skill to have included introns in the chimeric constructs that are described and claimed in the application.

7. I also understand that the Examiner is of the opinion that a skilled person would have derived from the Flavell, Metzlaff et al. and Stam et al. publications (or other unnamed contemporaneous publications relating to the field of co-suppression) that expression of target genes in a cell can be inhibited by the introduction of chimeric genes expressing sense and complimentary antisense sequences of the target gene (either from separate constructs or from the same construct) which can form a double stranded RNA molecule.

8. I further understand that the Examiner is of the opinion that a skilled person would then also have been motivated to build further on this alleged knowledge derived from the Flavell, Metzlaff et al. and Stam et al. publications and design constructs wherein complementary sense and antisense strands are expressed as inverted repeats in a single molecule. The Examiner has alleged that "*expression of a single contiguous self annealing construct would provide for more efficient self annealing compared to two separately expressed self annealing molecules, applying scientific logic to the teachings of Flavell, Metzlaff and Stam*"

9. I respectfully disagree with the Examiner for the reasons elaborated below. The publications by Flavell, Metzlaff et al. and Stam et al (or other contemporaneous publications related to the field of co-suppression) did not contemplate that double stranded RNA structures formed between antisense RNA and the sense mRNA could be a triggering agent in gene silencing. A person of ordinary skill in the art understanding the proposed models for gene silencing would not have included a sense and antisense RNA strand in one single molecule to obtain more efficient self annealing. This is because the proposed models and prevailing wisdom considered a antisense strand to be the operative gene-silencing triggering molecule. More efficient self annealing would sequester the antisense strand by base-pairing with the sense strand, which would be contrary to the mechanisms

proposed in the cited papers and generally understood at the time. I was a person of at least ordinary skill at the time and certainly would never have contemplated deliberately introducing complimentary sense and antisense sequence of a target gene which can form a double stranded RNA molecule to increase the efficiency of gene silencing based upon these papers or the general understanding in the art at the time.

10. The application describes methods for reducing the expression of a nucleic acid sequence of interest in eukaryotic cells, such as plant cells, by simultaneously providing the cells with chimeric genes encoding sense and anti sense RNA molecules targeted to the nucleic acid of interest (application page 1, line 5 – Example 2). The sense and antisense RNA molecules may be provided as one RNA molecule (application page 1, line 6- Example 1, page 38-40) that can be transcribed from a recombinant DNA and which is capable of forming an artificial hairpin structure. In its simplest form, the artificial hairpin RNA can be visualized as a double stranded RNA region formed between the complimentary sense and antisense region, and a loop (transcribed from the spacer region) connecting the sense and antisense region. The term "artificial hairpin RNA" refers to the requirement that the hairpin RNA is not naturally occurring in nature, because the sense and antisense regions as defined are not naturally occurring simultaneously in one RNA molecule, or the sense and antisense regions are separated by a spacer region which is heterologous with respect to the target gene, or because the hairpin is not contained within the RNA molecule it is normally associated with (Application page 22, lines 19-28).

11. The gene-silencing methods described in the application were found to be surprisingly more efficient than the gene-silencing methods described in the art, which used either antisense or sense (co-suppresion) regions. The higher efficiency of gene silencing can be ascertained by

a. the greater proportion of the population of transgenic plants in which chimeric genes encoding the artificial hairpin RNA molecules were introduced that exhibited a gene silencing effect; and/or

b. the greater proportion of the population of transgenic effect that exhibited a very pronounced gene silencing effect.

12. The application also disclosed that inclusion of an intron sequence in the chimeric DNA genes encoding the hairpin RNA molecule enhanced the efficiency of reduction of expression of the target nucleic acid to a surprisingly greater degree (application page 47; application page 23, lines 3-15).

13. This latter invention is recited in the current claims of the application. The claims are thus directed to a chimeric DNA comprising a promoter and a transcription termination and polyadenylation region operably linked to a DNA region, which when transcribed, yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, wherein one of the annealing RNA sequences of the hairpin RNA structure comprises a sense sequence identical to at least 20 consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest, and wherein the second of the annealing RNA sequences comprises an antisense sequence identical to at least 20 consecutive nucleotides of the complement of at least part of the nucleotide sequence of the nucleic acid of interest and wherein the DNA region further comprises an intron. Other claims are directed to eukaryotic cell and organisms, such as plants, containing such chimeric genes. The application further contains corresponding method claims, which are however currently withdrawn from consideration.

14. It is my opinion that the application described the invention in sufficient detail to demonstrate that the inventors had a complete conception of the invention and described it sufficiently such that a person of ordinary skill in the art would have understood what the invention was and how it was distinguished over the prior art. It is further my opinion that a person of ordinary skill in the art reading the application would have been able to make and use the invention consistent with the breadth of the claims.

15. As mentioned, such chimeric genes could be used to effect a more efficient silencing of the target gene's expression than the antisense or co-suppression methods described in the prior art. Antisense mediated gene-silencing is the earliest described gene-silencing. In the mid-nineties, it was generally assumed that the antisense RNA formed a double-stranded intermediate with its complementary mRNA resulting in either mRNA degradation by double-stranded RNA specific nucleases, inhibition of RNA processing, transport or translation.

16. In 1990, co-suppression was first described in purple flowered petunia plants genetically modified by the introduction of DNA containing a chalcone synthase coding sequence under the control of the strong CaMV35S. Chalcone synthase is a key enzyme in the flavonoid biosynthesis and therefore in pigment production. Surprising phenotypes were produced in that a very high proportion of the first family of primary transformants had flowers with white sectors, and the flowers of some plants were completely white. It was found that the white sectors of the flowers lacked anthocyanin pigment and contained very low levels of mRNA from both transgene and endogenous chalcone synthase A in petals. In 1995, I co-authored a chapter in a book entitled "Gene silencing in Higher Plants and Related Phenomena in other Eukaryotes" pages 43-58 (Ed. Peter Meyer- Springer Verlag) reviewing mechanisms and hypotheses for co-suppression of chalcone synthase, and other genes in transgenic plants (ANNEX II).

17. As described in that review, four kinds of hypotheses were put forward by the mid-nineties to explain the different gene-silencing phenomena.

18. In the first, inactivation of transcription is postulated due to the physical interaction in the nucleus of the duplicated but non-allelic sequences. Cycles of DNA-DNA or chromatin-chromatin interactions could leave the chromatin or methylation patterns of the participating genes in different states which could consequently interfere with the assembly of essential transcription complexes or the binding of the chromatin to the nuclear matrix.

19. The second hypothesis was based upon elevated competition between the increased number of genes for non-diffusible sequence-specific factors essential for ordered transcription or translation.

20. The third hypothesis focuses on post-transcriptional events. It postulated the degradation of the specific mRNAs due to the synthesis of homologous antisense RNAs in the cell, formation of double-stranded RNAs between the antisense RNA and mRNAs and recognition of the aberrant duplexes as substrates for a RNAse. These antisense RNAs could be made from an unknown promoter close to the transgene functioning in the appropriate orientation, possibly by read-through from a neighboring gene (as initially proposed by Grierson, 1991, TIBtech 9, 122-123) or by the action of RNA-dependent RNA polymerase on aberrantly accumulated mRNAs.

21. The fourth hypothesis postulated the inhibition of transcription and/or translation by feedback from a specific gene product that accumulates in aberrantly high concentrations in the transgenic plants, through a self-induced, autoregulated control system.

22. The review ends with a "Concluding perspective" section, wherein we indicated that from surveying the range of examples of gene silencing, it was clear that multiple mechanisms contribute to the observed phenotypes, and that is was desirable not to automatically lump all co-suppression phenomena into a homogeneous group and attempt to find a single mechanism for the observed gene silencing or lack of it. With regard to the co-suppression phenomenon associated with chalcone-synthase in petunia, we further indicated that the data accumulated to that date pointed to association of the phenomena with higher levels of mRNA synthesis and/or antisense RNA.

23. Thus, at that point, we favored the hypothesis that co-suppression was mediated through the involvement of an antisense molecule generated via a unknown mechanism from the sense RNA. The antisense RNA molecule could then form a dsRNA intermediate with the targeted mRNAs which were thus tagged for degradation.

24. In February 1998, Fire et al. published a surprising technique for inhibiting gene function in *C.elegans* by injecting double stranded RNAs corresponding to a target gene in the small worms (Fire et al., 1998; Nature 391, pages 806-811) Although intriguing and efficient, the double stranded RNA hypothesis posed a puzzle of how to reconcile all previously obtained data regarding antisense and sense mediated gene-silencing. As indicated in the editorial comment by Wagner and Stam in the same Nature issue (Wagner and Stam, Nature 391, pages 744-745, ANNEX III), a lot of questions remained unresolved: " *Fire and colleagues have uncovered a complex and intriguing mode of regulation in C. elegans....Does a similar phenomenon exist in other organisms? What would happen if transgenic animals or plants were generated expressing both the sense and antisense strands of a transgene?*"

25. It is currently understood that dsRNA is a gene-silencing triggering molecule, (which may be generated during viral replication, or by synthesizing a complementary strand on aberrant sense or antisense RNA molecules through the action of a RNA dependent RNA

polymerase). The dsRNA molecule is cleaved by a dsRNA specific RNAse (called Dicer or Dicer-like) into smaller dsRNA molecules of 21-24 nt long. The "antisense" strands of these smaller dsRNA molecules are loaded onto a RISC protein complex and act as a guidance molecule to direct the degradation of the corresponding mRNA.

26. I will now specifically address the publications relied on in the Office Action. Starting with Flavell (1994), I disagree with the Examiner's analysis (Office action at page 6) that this publication discloses "*plants, eukaryotic cells and chimeric DNA comprising an operable promoter, transcription termination and polyadenylation region and further comprising a DNA region encoding a region capable of forming a double stranded RNA stem by base pairing between regions with a sense and antisense nucleotide sequence, which sense nucleotide sequence includes at least 10 consecutive nucleotides having 100% sequence identity with at least 10 consecutive nucleotides having 100% sequence identity with said at least 10 consecutive nucleotides of the sense sequence*" I cannot find either at the indicated passages or anywhere else a disclosure of such structures. Moreover, this analysis by the Examiners seems to be contradictory to the Examiner's conclusion that the" *primary references of Flavell [...] [does] not teach double stranded hairpin constructs in their inverted repeats*" (Office action at page 7, last sentence).

27. Flavell reviews four categories of explanation of the gene-silencing phenomenon that were proposed around 1994 including

a. Adaptation of an epigenetic state that affects gene expression

b. Competition between genes for nondiffusable factors essential for ordered transcription or translation

c. Production of unintended antisense formation and the degradation of mRNA sense-antisense duplexes.

d. Accumulation of higher levels of a specific RNA and degradation of all this mRNA species by some unknown mechanism (page 3492, first column).

Of these 4 categories, only the third one involves the formation of a double stranded RNA intermediate, however this duplex RNA is proposed to form between the produced antisense RNA and the sense messenger RNA and trigger the degradation of the sense messenger RNA. Note that in this model the double stranded RNA molecule is thus not made between an antisense RNA region and an sense RNA region, different from the target mRNA to be degraded. According to this model, the antisense RNA is the pivotal molecule in gene-silencing and may be introduced intentionally or unintentionally (via transcription under control of promoters outside the transgene or through the action of RNA dependent RNA polymerase on aberrant RNA templates). In view of this model, it would not be logical to enhance the efficiency of gene silencing by simultaneous introduction of a sense and antisense RNA molecule capable of forming a duplex RNA with each other, since this sense RNA molecule would "compete" with the targeted mRNA molecule for duplex formation with the active antisense molecule triggering the gene-silencing phenomenon. The competition would even be more severe if the introduced sense and antisense RNA would be present in one molecule, as such intramolecular duplex formation would be favored over intermolecular duplex formation.

28. Turning to Stam et al., I also disagree with the Examiner's analysis (Office action at page 7) that this publication discloses "*plants, eukaryotic cells and chimeric DNA comprising an operable promoter, transcription termination and polyadenylation region and further comprising a DNA region encoding a region capable of forming an artifical hairpin RNA structure with a double stranded RNA stem by base pairing between regions with a sense and antisense nucleotide sequence, which sense nucleotide sequence includes at least 10 consecutive nucleotides having 100% sequence identity with at least 10 consecutive nucleotides having 100% sequence identity with said at least 10 consecutive nucleotides of the sense sequence*" . Again, I cannot find at the indicated passages or anywhere else a disclosure of such structures.

29. Stam et al. review potential models to explain posttranscriptional gene silencing. As indicated in the legend of Figure 1, "the key features considered are: the production of aberrant transcripts, the activity of host encoded RNA-directed RNA polymerase and the production of complementary RNA (cRNA or antisense RNA)". Thus, as elaborated with regard to Flavell (paragraph 11), Stam et al attribute a central role in gene

silencing to the antisense RNA molecule. The mentioned repeat structures are not within a single gene (as contemplated by the Application) but are with regard to entire transgenes which can be in inverted and direct repeat. According to Stam et al. (page 8) such a multicopy locus is prone to deliver the hypothetical aberrant RNA which through the action of RdRP could yield complementary RNAs which can hybridize to the (sense) mRNA and be degraded. Again, as elaborated above for Flavell, Stam et al. disclose that double stranded RNA structures formed between antisense RNA and the sense mRNA can be involved as an intermediate in gene silencing but are not described as a triggering agent.

30. Metzlaff et al. is a publication that I co-authored with Flavell and others. It reports work performed in the labs of Richard Flavell. In particular, we analyzed the presence of shorter poly(A)- and poly(A)+ RNAs in petunia plants where the chalconsynthase expression is reduced through the introduction of a sense chalconsynthase gene under control of a CaMV 35S promoter, resulting in white flowers or flowers with white sectors. As explained above in paragraph 11, our working hypothesis was that sense RNA mediated co-suppression involved generation of an antisense RNA which could hybridize with the messenger RNA leading to degradation of both endogenous and transgene chalcone synthase mRNA. The model presented in Metzlaff et al. also involves complementary RNA, but in the case of chalconsynthase RNA the complimentary RNA is inherent in the sense transcript (see discussion last sentence) as the *chs* mRNA naturally contains complementary sequences (43 bp which are 80% complementary and are located at the 3' end of coding sequence of *chs* gene and in the 3'untranslated region). It will be clear that the last sentence in the abstract concerning the presentation of "*a model involving cycles of RNA-RNA pairing between complementary sequences followed by endonucleolytic RNA cleavages*" refers to the model schematically presented in Figure 7, where a duplex RNA molecule is formed between the aberrant RNA *chs* molecules and the complementary region elsewhere in the chs mRNA to initiate endonucleolytic cleavage resulting in further aberrant RNA chs derived molecules, perpetuating the cycle of duplex formation with the mRNA and cleaving thereof.

31. In conclusion, Flavell, Stam and Metzlaff all emphasize the importance of the complementary RNA/antisense RNA as the central effector molecule in gene silencing. I

reiterate that accordingly it would not be logical to enhance the efficiency of gene silencing by simultaneous introduction of a sense and antisense RNA molecule capable of forming a duplex RNA with each other, since this sense RNA molecule would compete with the targeted mRNA molecule for duplex formation with the active antisense molecule triggering the gene-silencing phenomenon. The competition would even be more severe if the introduced sense and antisense RNA would be present in one molecule, as such intramolecular duplex formation would be favored over intermolecular duplex formation.

32. It is therefore my opinion that it would **not** have been obvious to one of ordinary skill in the art to derive from the Flavell, Metzlaff et al. and Stam et al. publications (or other contemporaneous publications in the field of co-suppression in plants) that expression of target genes in a cell can be inhibited by the introduction of chimeric genes expressing sense and complimentary antisense sequences of the target gene (either from separate constructs or from the same construct) which can form a double stranded RNA molecule.

33. I hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 18/03/2009

Michael Metzlaff

ANNEX I

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Curriculum Vitae: Michael Metzlaff

2008 -	Research Liaison Manager Bayer BioScience Management of Research Collaborations between Bayer BioScience and Academia
2003-2008	Group Leader and Program Leader at Bayer BioScience N.V. Gent, Belgium Administrative tasks: steering and coordination of Bayer's Crop Productivity Research Own research: molecular genetics of abiotic stress tolerance, gene silencing/RNAi
1999–02	Senior Scientist at Plant Genetic Systems N.V., Aventis CropScience N.V. and Bayer BioScience N.V. carrying out research in virus- and transgene-induced gene silencing
1993-98	Senior Scientist at John Innes Centre, Norwich, UK, Department of Genetics working together with Dick Flavell in the field of plant gene silencing
1987-92	Lecturer in Genetics at Martin-Luther-University Halle, Germany Teaching: Plant Molecular Genetics, Genetic Engineering Research: organelle-nucleus interactions in plants, molecular organization of non-coding repetitive sequences, molecular marker-assisted plant breeding
1987	Habilitation (Dr.rer.nat.habil.) in Plant Genetics at Martin-Luther-University
1983-86	Group Leader at Martin-Luther-University, Department of Genetics Research: molecular organization of nuclear and organelle genomes in higher plants of the genera *Pelargonium, Antirrhinum, Lycopersicon, Beta, Triticum* and *Secale*
1980-83	PhD student at Martin-Luther-University, Department of Genetics Topic of thesis: chloroplast DNA variation in the genus *Pelargonium*
1975-80	Studies in Biology at Martin-Luther-University Halle, Germany
1954	Born in Genthin, Germany on November 26
Publications:	- approx. 50 publications in peer-reviewed scientific journals - contributions to several scientific books - numerous invited talks at scientific meetings - regular reviewing of thesis, manuscripts and research projects at national and international level

Publication list Michael Metzlaff

1980

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1981

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1982

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1983

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ANNEX II

Current Topics in Microbiology and Immunology

197

Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes

Edited by P. Meyer

With 17 Figures

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Cover Illustration:
The front page shows three different examples of gene silencing phenomena in plants: (1) Silencing of a transgenic pigmentation marker in petunia flowers due to DNA methylation (background photo, provided by Iris Heidmann). See p. 15 for further details. (2) Inhibition of tomato fruit ripening by antisense technology (photos in the upper two panels, provided by Don Grierson). Wild-type tomatoes (right) and antisense transformants (left) are shown. See p. 77 for further details. (3) Silencing of anthocyanin pigmentation in maize anthers by paramutation at the P1 locus (photos in the lower two panels, provided by Garth Patterson). The P1-Rh phenotype (right) and the P1'-mah phenotype (left) are shown. See p. 121 for further details.

The photo on the back page shows examples of flower phenotypes derived from co-suppression of a gene of the pigmentation pathway in petunia (photo provided by Richard Flavell). See p. 43 for further details.

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Developmental Regulation of Co-suppression In *Petunia hybrida*

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1 Introduction

The gene silencing phenomenon to be discussed here, initially termed "co-suppression" (Napoli et al. 1990; Van der Krol et al. 1990; Jorgensen 1990), was observed in purple-flowered petunia plants genetically modified by the introduction of DNA containing a chalcone synthase coding sequence under the control of the strong CaMV 35S promoter and the 3' end from the nopaline synthase gene of *Agrobacterium*. The selectable marker gene consisting of the coding sequence for neomycin phosphotransferase under the control of nopaline synthase promoter and with the 3' end from the octopine synthase gene was also inserted on the same T-DNA. These genes were introduced into petunia cells via the transferred portion of the Ti plasmid of *Agrobacterium tumefaciens* (i.e. the T-DNA).

Chalcone synthase is a key enzyme in flavonoid biosynthesis and, therefore, in pigment production. These pigments are synthesized intensely in the epidermis of flower petals, but also to lesser extents in many other parts of the plant including the anthers. Pigment production is cell-type specific. Chalcone synthase gene expression is transcriptionally regulated but separate post-transcriptional effects have been described that influence the pigmentation pattern in flowers (Mol et al. 1983). In petunia, chalcone synthases are encoded by a gene family (Koes et al. 1989), and the cDNA used to create the new transgene was

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from the chalcone synthase A (CHS A) allele responsible for most of the chalcone synthase activity in petals (Koes et al. 1989). Surprising phenotypes were produced in that a very high proportion of the first family of primary transformants had flowers with white sectors, and the flowers of some plants were completely white (Napoli et al. 1990). This was interpreted to imply that the introduction of a new chalcone synthase gene had caused the loss of most or all chalcone synthase activity from the inserted transgene and the endogenous chalcone synthase genes in the white petal sectors. This interpretation was confirmed by the correlation between the lack of anthocyanin pigment and the very low levels of mRNA from both transgene and endogenous CHS A in petals (Napoli et al. 1990; Van der Krol et al. 1990). This suppression of both kinds of homologous gene was the reason for using the term co-suppression to describe the phenomenon (Napoli et al. 1990). Subsequent analyses of large numbers of transformants and their progeny from the selfing and backcrossing of selected transgenic lines have revealed numerous important features about the co-suppression phenomenon (Jorgensen 1993b, 1994, and unpublished results).

2 Co-suppression and Plant Development

The flower phenotypes showing co-suppression have been classified on the basis of the position and extent of pigmentation in the flowers (Jorgensen 1993a,b). This classification is meaningful because phenotypes are characteristic for particular transformants even though new variants may arise, as described below. Some of the phenotypes are shown in Fig 1. They range from completely white where pigment production is suppressed in all parts of the flower—tube, corolla and anthers—to other patterns where the white segments are small. In one pattern the pigmentless sector is confined to the tube and the anthers, but frequently extends just outside the tube and to a greater extent on the lower petals. In others pigment loss occurs in small sectors along the veins and/or petal tips. In another pattern, pigment loss is orientated along the edges of the petals. The areas without pigment can be much larger in some phenotypes (Napoli et al. 1990). In yet another series of plants, the white sectors are small and dispersed across the flower in complex patterns. All these patterns point to inherent features of flower development that are revealed by the transgene. The cells which lack pigment are not simply clonally related. Instead it appears that cells occupying certain locations in the floral meristem with respect to architectural features of petal shape such as lines of symmetry, respond similarly (but not identically) from petal to petal to the presence of the transgene, and these responses are different from those of other cells in other positions. The pattern boundaries are coincident in the upper and lower epidermis. Thus pattern formation may also require intercellular communication.

The untransformed parent plants show no evidence of such pigmentation patterns, though other varieties do (Red Star and Velvet Picotee; Mol et al. 1983;

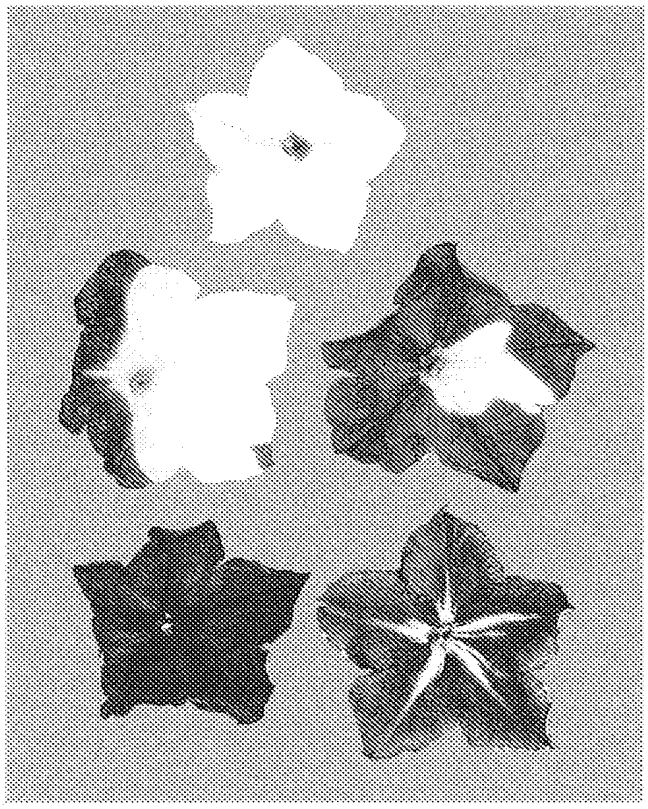

Fig. 1. Flower patterns resulting from insertion of the transgene consisting of the coding sequence of CHS A under the control of the CaMV 35S promoter (see colour version of the figure on the backcover). The untransformed parent has only purple flowers. Phenotypes from top middle round to bottom left display: extensive co-suppression emanating from lower petal junctions, complete co-suppression, co-suppression from lower petal junctions, co-suppression along petal veins

Van Blokland 1994). From the principles of flower design, one can assume that the architectural basis of the patterns is not caused by the transgene but is an inherent feature of flower development. However, elements of this feature somehow interact with the transgene or its product to produce the observed patterns, and different versions (states) of the transgene interact differently to create the different patterns (Jorgensen 1993a,b).

The observation that many transgenic plants display a characteristically patterned flower phenotype, based on the patterns of co-suppression, implies that the 'state' of the transgene is somatically inherited. When the meiotic inheritance of transgene effects on flower phenotypes was examined (Jorgensen 1993b and unpublished), several outcomes were noted. In many cases, the phenotype bred true and is thus germinally stable. In other plants examined, a new range of somatically inherited phenotypes was observed. For example, from

a backcross between a white transformant containing two tandem copies of the new genes and its untransformed parent, many phenotypes were obtained including fully purple, fully white and various patterned types. In these cases the phenotype based on the floral positions of cells showing co-suppression is germinally unstable and the transgene presumably alters its state. Thus it can be concluded that a given transgene can exist in different epiallelic states, and these states can change during meiosis or early embryonic development (Jorgensen 1993b). Occasionally a lateral branch emerges that displays a different flower phenotype with more or less pigmentation, and the variation is inherited, implying that a change has occurred in the L2 layer of cells in the flowers (Jorgensen 1994 and personal communication).

Petunias produce flowering branches from organised groups of cells (meristems) in the axils of leaves or on the flanks of meristems. A genetically different branch results if the group of cells in the meristem flank becomes modified. Occasionally single variant flowers, gradients of phenotypic change as a branch ages, and simultaneous changes in different branches have been noted (Jorgensen 1994) implying that changes can occur in any floral meristem. Because the inherited L2 layer and the L1 layer in which epidermal pigment is produced are separate developmental lineages of cells, it is reasonable to consider the possibility that the changes in transgene state behind pattern changes occur in many cells of a meristem essentially simultaneously.

The remainder of this chapter deals with the origins of the pigmentless phenotype created by the insertion of the CHS A coding sequence under the control of the CaMV 35S promoter.

3 Hypotheses to Explain Gene Silencing

Numerous examples are known, in at least six plant species, where gene inactivations results from the introduction of additional homologous sequences. These have been reviewed elsewhere (Jorgensen 1990, 1991, 1992; Mol et al. 1991; Kooter and Mol 1993; Matzke and Matzke 1993; Matzke et al. 1993; Assaad et al. 1993; Vaucheret 1993; Goring et al. 1991; Meyer et al. 1993; Grierson et al. 1991; Flavell 1994; Hart et al. 1992; Meins 1989; Meins and Kunz 1994), and in other chapters in this book (for example see Hamilton et al. and de Lange et al., this volume). They will not therefore be discussed extensively here. However, it should be noted that no single mechanism can explain the variety of examples where loss of gene expression has occurred.

Four kinds of hypotheses have been put forward to explain the diversity of gene silencing phenomena. In the first, inactivation of transcription is postulated due to the physical interaction (ectopic pairing) in the nucleus of the duplicated but non-allelic sequences (loci). Cycles of DNA–DNA or chromatin–chromatin interactions (see Fig. 2) could leave the chromatin structure or methylation patterns of the participating genes in different states which could consequently interfere with

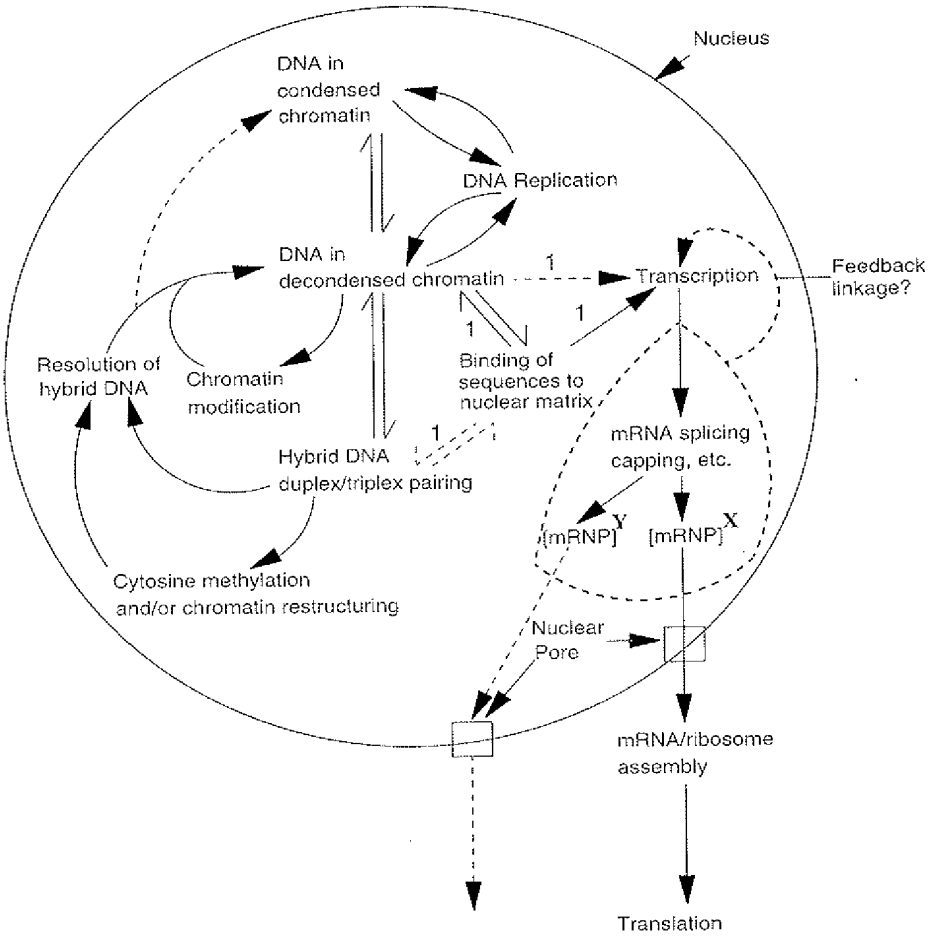

Fig. 2. Cellular processes relevant to models for gene silencing. The network of pre-transcriptional events illustrates how the structure of decondensed chromatin, the substrate for transcription, can be modified by various sorts of changes including cytosine methylation and interactions with homologues including transgenes. The changes could modify decondensed chromatin such that it does not bind properly to the nuclear matrix or bind transcription complexes efficiently. After "normal" transcription mRNA is processed, capped and polydenylated in messenger RNA nuclear protein particles $[mRNP]^X$ which are then exported from the nucleus and the mRNA traslated on ribosomes. Where gene silencing is post-transcriptional, transgene mRNA processing, splicing, capping or polyadenylation could be aberrant, thereby leading to synthesis of aberrant particles $[mRNP]^Y$. These might not be translated efficiently and may be substrates for RNases. They may also be substrates for antisense RNA formation. In any event they do not give rise to protein product. There is the possibility of aberrant mRNA production influencing transcription. Further details are described in the text

the assembly of essential transcription complexes or the binding of the chromatin to the nuclear matrix. These processes are labelled 1 in Fig. 2. There is no direct evidence for such interactions occurring in plants, but precedents come from studies on fungi and *Drosophila*. In *Neurospora* and *Ascobolus*, DNA homology-searching processes and hybrid DNA formation have been inferred from the inactivation of duplicated sequences via cytosine methylation in premeiotic cells (Selker 1990; Foss and Selker 1991; Rhounim et al. 1992; Faugeron et al. 1990). In yeast, equivalent frequencies of allelic and ectopic meiotic recombination have also been taken to imply the existence of efficient, generalised, DNA sequence homology searching processes (Haber et al. 1991). In *Drosophila* there are many examples where expression of a gene is influenced by "sensing" the presence of another specific gene after some kind of localised somatic chromosome pairing. The pairing could be mediated via DNA, RNA or transcription complexes. The consequentially altered chromatin, sometimes heterochromatic, state created following the interlocus interactions can be clonally inherited when not disturbed by other events (Tartof and Henikoff 1991; Henikoff 1992; Pirotta 1990; Wu 1993; Paro 1990).

The second hypothesis is based upon elevated competition between the increased number of genes for non-diffusible sequence-specific factors essential for ordered transcription or translation.

The third hypothesis focuses on post-transcriptional events. It postulates the degradation of the specific mRNAs due to the synthesis of homologous antisense RNAs in the cell, formation of double-stranded RNAs between the antisense RNA and mRNAs and recognition of the aberrant duplexes as substrates for a RNase. Mutual inactivation of homologous mRNAs can often be achieved by the introduction of antisense gene. Double-stranded RNAs may also inhibit translation if they are formed in the cytoplasm (Temple et al. 1993; Cornelissen and Vandewiele 1989). Evidence for the existence of dsRNA in plants is however very weak (Grierson et al. 1991; Jorgensen 1991; Mol et al. 1991). These antisense RNAs could be made from an unknown promoter close to the transgene functioning in the appropriate orientation, possibly by readthrough from a neighbouring gene or by the action of RNA-dependent RNA polymerase on aberrantly accumulated mRNAs (Lindbo et al. 1993; Flavell 1994). This latter enzyme exists in plant cells.

The fourth hypothesis postulates the inhibition of transcription and/or translation by feedback from a specific gene product that accumulates in aberrantly high concentrations in the transgenic plants. This would constitute a self-induced, autoregulated control system (Hart et al. 1992, Meins 1989; Meins and Kunz 1994).

4 Mechanisms and Hypotheses for Co-suppression of Chalcone Synthase in Transgenic Petunias

We now consider the petunia chalcone synthase case in the light of these hypotheses. Throughout this discussion, it is relevant to bear in mind that not all transgenic plants containing CHS A transgenes display co-suppression in the petals, and that in some plants only specific segments of petals show co-suppression. Also, it is important to remember that inherited somatic and meiotic changes can occur to influence the extent to which co-suppression is observed.

In the process of making the transgenic plants, it can be expected that different numbers of T-DNAs become stably inserted into different petunia plants and display different structures. Tandem arrays of T-DNAs are common, as are copies inverted with respect to one another. Genetically unlinked T-DNAs also accumulate. Thus plants with different numbers of active genes are likely to be produced, as noted in other studies on transgenic plants (Hobbs et al. 1993; Assaad et al. 1993; Scheid et al. 1991; Linn et al. 1990). It will be important to investigate thoroughly whether the structure of T-DNA inserts influences the extent of co-suppression and the kinds of flower pattern produced. Van Blokland (1994) has concluded that phenotypic effects of CHS A transgenes are correlated with the presence of inverted repeats of T-DNA.

Where there are multiple copies of the chalcone synthase transgene then the copies might interact (see Fig. 2) to silence transcription of one another and the endogenous CHS A genes. Such silencing has been recorded for several sorts of transgenes (Peach and Velten 1991; Assaad et al. 1993; Hobbs et al. 1990, 1993; Elkind et al. 1990; Linn et al. 1990; Matzke et al. 1994b; Vaucheret 1993). Inverted repeats seem to be more frequently associated with transcriptional silencing (Hobbs et al. 1993). How such physical interactions occur is unknown, but they may be the means whereby one or more of the duplicated sequences gain some methylated cytosines. No evidence for silenced CHS A genes becoming routinely methylated has yet been obtained in investigations of several sites within the coding sequences and promoters.

There is evidence, however, that in some transgenic petunias, CHS A transcription is not blocked in petal cells showing loss of pigment. Run-on transcription assays on nuclei from isolated purple and white petal sectors from the same plant show similar levels of CHS A transgene and endogenous CHS A transcription (Van Blokland 1994). Furthermore, similar levels of unprocessed nuclear endogenous CHS A transcripts have been detected in flowers of some co-suppressed and non co-suppressed variant plants in our laboratory and in that of Mol and co-workers. The levels of RNA transcribed in isolated petal nuclei are not correlated with the extent of chalcone synthase suppression (Van Blokland 1994; Kooter and Mol 1993; Mol et al. 1991). These details are reviewed in another chapter in this book (de Lange et al., this volume). We have also found in some plants that white flower sectors retain high levels of CHS A RNA, making it likely that post-transcriptional losses of functional mRNAs are the cause of or a

major contributor to the co-suppression phenotype. Studies of inactivation of some other transgenes in plants have also concluded that the inactivation is post-transcriptional (Smith et al. 1990a; de Carvalho et al. 1992; Bate et al. 1992; Meins and Kunz 1994).

In plants where transcription of the CHS A transgenes is not blocked but steady state functional mRNA levels are very low, then a major cause of co-suppression could be accumulation of excess levels of antisense RNAs to the CHS A mRNA, double-stranded RNA formation and degradation of the duplex RNA (third hypothesis above). The presence of antisense RNA to chalcone synthase has been investigated in transgenic petal tissues differing in co-suppression, i.e. purple and white. Of the particular variants studied by us, most were derived from the same transgenic parent and possess two copies of the transgene in inverted orientation. Reverse transcriptase and primers specific for antisense RNA were used to make DNA copies of RNA in RNA extracts isolated from white or purple sectors. Antisense chalcone synthase RNAs were found in both white and purple flower sectors but only in transgenic plants. It is, therefore, concluded that the antisense RNAs are due to the transgene. The finding that antisense chalcone synthase RNAs are in both white and purple sectors suggest that if antisense RNA is essential for the loss of mRNA and gene expression in this genotype, it is clearly insufficient. Similar conclusions have been drawn by Mol and co-workers (de Lange et al, this volume; Van Blokland 1994) who used other assays to detect antisense RNA.

How is antisense RNA produced from the transgene, what is its structure, and how does it function? These important questions still have to be examined experimentally. It will be necessary to examine many different transgenic plants with different numbers and kinds of transgene structures since it is not clear how antisense RNA could be produced so efficiently in all transformants (Jorgensen 1991). Where antisense RNA is not transcribed from defined genomic promoters it could be formed by an RNA-dependent RNA polymerase using sense mRNA as template (Lindbo et al. 1993; Flavell 1994).

On simple considerations of how antisense RNA interferes with sense mRNA, it would be assumed that the higher the antisense to sense RNA ratio, the more efficient would be the loss of sense gene expression. Some data in plants to support this have been produced (Smith et al. 1990b; Hamilton et al. 1990; Cannon et al. 1990; Robert et al. 1989; Van der Meer et al. 1992). However, there are many reports of discrepancies between the relative levels of antisense RNA transcripts and loss of sense gene expression (reviewed in de Lange, this volume; Cannon et al. 1990; Stockhaus et al. 1990; Van der Krol et al. 1988). Van Blokland (1994) found in petunias transgenic for chalcone synthase that antisense transcription could be high in the absence of co-suppression or vice versa. If antisense RNA is the cause of degradation of CHS A mRNAs, but overall steady state or transcription levels of antisense mRNA do not correlate with co-suppression it must be a small fraction of the antisense RNA that is critical, and this fraction must have efficient access to the unprocessed primary RNA transcripts or mRNAs formed after processing, capping and poly-A tail additions. This implies

that the variation in co-suppression in transgenic plants that makes antisense and sense RNAs could involve variation in the accessibility of antisense and sense RNAs to each other in the nucleus or the cytoplasm (see later).

Post-transcriptional loss of CHS A gene expression and pigment production could, alternatively, be due to the accumulation of excess levels of CHS mRNA and the consequential induction of an mRNA-specific process, that is able to catalyse the inactivation and/or degradation of transgene and endogenous CHS A mRNAs (fourth hypothesis above). This hypothesis includes the notion of critical localised threshold levels of mRNA in a cell. Where mRNA levels are below the threshold, purple pigment is produced; in contrast, when the level is exceeded, active mRNA is lost and no pigment is produced. There are several predictions from this model.

1. In plants where all flower epidermal cells lack pigment, the threshold mRNA concentrations are likely to have been exceeded in all cells of the plant. This is as observed in many white flowered plants where levels of transgene mRNA are very low in stems and leaves, as well as flowers (Napoli et al. 1990; this laboratory, unpublished).
2. In plants which have purple flowers with white sectors, the levels of localised active mRNA is likely to be higher than that found in plants that make only purple flowers. This is because in such plants small increases in the level of mRNA accumulated would exceed the threshold more readily and thus lead to the pigment loss. Some evidence has been gained to support this hypothesis in that the levels of transgene CHS mRNA accumulating in leaves of transgenic plants with purple and white flowers is greater than in leaves of transgenic plants forming only purple flowers (unpublished results).

If localised mRNA concentrations are the determinant of the post-transcriptional trigger for co-suppression, then the critical parameters affecting co-suppression would be the rate of transgene transcription and/or changes in the efficiency (rate) of mRNA transport through the nucleus, of export through the nuclear envelope, of binding to the ribosome and of translation. Changes in flower pigment production due to different levels of translatable CHS A mRNA could therefore come about through (a) pre-transcriptional events including changes in the levels of transcription factors, restructuring of chromatin, (and/or) changed cytosine methylation to affect the affinity of the transgenes for transcription complexes as noted earlier (see Fig. 2), and/or (b) changes in the rate of mRNA transport, etc. The latter could result from the transgene altering its position in the nucleus with respect to nuclear transport channels and the supply of protein components of the mRNPs essential for correct mRNA processing, transport and export (Flavell 1994). Furthermore, aberrantly high levels of mRNA in the nucleus might lead to mRNP particles with a different complement of proteins (Wolffe 1994) from those formed when the CHS genes are optimally transcribed for mRNA processing and transport and when nuclear mRNA levels are much lower (see Fig. 2). Such modified mRNPs might not make the mRNAs available for translation.

A growing number of proteins are known that bind to mRNA and prevent translation as part of specific regulatory mechanisms. The studies on the ubiquitous Y box proteins and the FRG Y proteins of Xenopus oocytes, in particular, are interesting (Wolffe 1994). These proteins bind to mRNAs and inhibit their translation (Bouvet and Wolffe 1994). They will bind to a range of double and single stranded DNAs and RNAs including specific Y box sequences in gene promoters (reviewed in Wolffe 1994). They also stimulate mRNA synthesis, but not necessarily by binding to the promoter motifs. FRG Y2 (a predominantly nuclear Y box protein) has been immunolocalised to nascent transcripts on lampbrush chromosomes (Sommerville et al. 1993). These observations are consistent with a direct role for FRG Y2 in packaging mRNA in the nucleus and for somehow linking the competence of the mRNA for translation with its transcription (Bouvet and Wolffe 1994). Recruitment of some other heterogeneous nuclear (hn) RNP proteins on to pre-mRNA is also dependent on transcription (Pinal-Roma and Dreyfuss 1992), and there is a growing list of eukaryotic proteins having dual roles in the transcription and translation processes (reviewed in Bouvet and Wolffe 1994).

If protein-based regulatory systems linking transcription, mRNA packaging and mRNA translatability are present in plants, then they might be responsible for post-transcriptional loss of gene expression following "aberrant" mRNA synthesis from the CHS A transgenes. It is also possible that they could lead to shut down of transcription. This whole area of nuclear biology needs to be explored in the context of understanding how aberrant active CHS A transgenes can promote loss of pigment production in flower petals. While it is possible to imagine how mRNA from an aberrantly located transgene might be sequestered into an inactive mRNP structure, how would this affect mRNAs from the endogenous CHS A genes? Perhaps the protein-mediated regulatory systems could also provoke cross-talk between homologous mRNP complexes and sequester all CHS A RNAs into aberrant mRNPs.

If excess CHS mRNAs accumulate due to higher rates of transcription or low rates of mRNP maturation in the nucleus and/or translation, how are they degraded? This could result from the aberrant mRNPs being recognised by RNases and the RNAs consequently degraded (Sachs 1993; Sullivan and Green 1993). Alternatively, antisense RNA could be produced on the accumulated mRNA templates by RNA-dependent RNA polymerase, and these double-stranded RNA structures are subsequently degraded. Cycles of RNA production of both antisense and sense RNAs could emerge from this process to provide an autocatalytic system for the production ds RNAs. Any such antisense RNAs could, of course, explain loss of both transgene and endogenous CHS A mRNAs.

5 Concluding Perspective

From surveying the range of examples of gene silencing, it is clear that multiple mechanisms contribute to the observed phenotypes and in some examples

mechanisms resulting in inhibition of transcription are major determinants, while in others post-transcriptional events occur. This diversity of mechanism may also appear between plants genetically altered by insertion of the same or related transgenes, but at different sites, in different arrangements and with different effective promoter strengths. Thus it is desirable not to automatically lump all petunias involving CHS A transgenes into a homogeneous group and attempt to find a single mechanism for the observed gene silencing or lack of it.

The scenario described above for the post-transcriptional control of chalcone synthase silencing may also be combined with, or lead to, variable patterns of transcription silencing in different genotypes. The data accumulated to date point to association of the phenomena with higher levels of mRNA synthesis and/or antisense RNA, and it has been argued that there are probably more than one cellular pool or RNP package for each of these molecules. The fact that sense mRNAs, may exist in different RNP pools and packages implies that we need to look for the different structural forms that may have different stabilities and opportunities to associate with ribosomes and be translated. Similarly we need to investigate whether different antisense RNPs exist.

If only one of the antisense RNP pools is available to interact with only one of the classes of sense mRNP, then the interacting classes are likely to be degraded in co-suppressed tissues, while other classes or pools might not be. Such discoveries might help explain the lack of correlation between antisense RNA to sense mRNA ratios and co-suppression phenotypes in different plants and tissues.

Hypotheses that propose the formation of different mRNP packages from active transgenes in aberrant nuclear positions and the production of pools of antisense RNA in some cases offer the following sorts of explanations for the origins of purple and white flower sectors: In transgenic plants where CHS A transgene transcripts are efficiently processed, packaged and exported then aberrant mRNPs would not accumulate and so co-suppression would not occur. Such plants would have purple flowers. If antisense RNPs were produced in such plants, the antisense RNA might not be accessible to the sense mRNPs and so the flowers would be purple. If, however, transgene mRNA were processed, packaged and exported inefficiently, due to the location or other features of transgene chromatin, then critical levels of nuclear mRNA would be exceeded, packaging could be aberrant and a different mRNP structure for all CHS A mRNAs might result. Messenger RNA in this structure might not be translated, or might be accessible to RNases and antisense RNAs or to RNA-dependent RNA polymerase that makes antisense RNA. Any of these would result in the formation of white flowers.

These ideas are testable and imply that switches in pigment production during transgenic CHS A plant development could result from (a) a change in nuclear position of the transgene; (b) a change in transcription rates (these would constitute inherited changes in state of a transgene); (c) a change in cell physiology influencing nuclear processing, RNA packaging, export and mRNA translation rates; and/or (d) a change in antisense RNA synthesis. Variation in such parameters would not be surprising during meristem development, growth in

different environments or during specific developmental phases. The patterns in flowers are presumably due to similar changes.

The developmental changes influencing the nuclear metabolism of specific clusters of cells in floral meristems and floral tissues are unknown. However, analyses of the CaMV 35S promoter have revealed that it contains multiple elements that respond differently in different petunia floral tissues, leading to differential transcription (Benfey and Chua 1989; Benfey et al. 1989). Thus the enhanced probability that co-suppression occurs in the flower tube and veins in some transgenic genotypes containing CHS A under the control of the CaMV 35S promoter or in other regions in other genotypes could be due to differential interactions between the promoter and enhancer structures of the promoter and the transcription factor concentrations present in different sectors of the meristem and floral tissues.

In conclusion, it is clear that studies into the origins of co-suppression and gene silencing in general will teach us many new features of cell biology and the control of gene expression. Furthermore, because of the wish to create agriculturally novel transgenic plants, understanding how active transgenes can lead to gene silencing is of considerable commercial interest.

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ANNEX III

named after the place of its invention in Eugene, Oregon. The model is a set of three partial differential equations that describe the reaction–diffusion process. Showalter and colleagues added a term to account for the photosensitive generation of bromide ions, and predict wave propagation patterns remarkably similar to those observed in the experiment. Before this experiment, STSR had been studied only theoretically or by numerical or electronic simulation in one-dimensional sets of coupled[6,7] and uncoupled[8] elements, and in two-dimensional arrays of threshold elements[9]. But those 2D simulations, in spite of their simplicity, mimic all the features of the present experiment.

The implications of the present experiment extend far beyond chemical dynamics. Spiral waves, spontaneously generated by noise, have also been simulated with the Oregonator (Fig. 1b). They are strikingly similar to recent observations of noise-initiated and sustained long-range coherent waves of calcium ions in cultured brain tissue[10] (Fig. 1c) indicating a similar underlying dynamical process. The possibility that calcium waves transmit or coordinate information over centimetre distances in glial cell networks (that is, in the brain) has already been suggested, but the role of noise remained obscure. Now that noise-sustained spiral waves have been observed in a well characterized chemical system, we can speculate that spatiotemporal noise may be an important feature of the brain's working. □

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Functional genomics

Double-stranded RNA poses puzzle

Richard W. Wagner and Lin Sun

The human genome is predicted to contain between 50,000 and 100,000 genes[1]. To work out what these genes do, an array of techniques is needed to evaluate the protein–protein interactions and biochemical pathways of any gene product. The nematode worm *Caenorhabditis elegans* is an excellent system for such studies because of its well-understood genetics and development, evolutionary conservation to human genes, small genome size and relatively short life cycle. The 100-megabase-pair genome will be completely sequenced this year, and a total of 17,000 genes have been predicted, many with human counterparts. Approaches used to manipulate gene expression in *C. elegans* include transposon-mediated deletion[2], antisense inhibition[3] and direct isolation of deletions after mutagenesis[4,5]. Although these methods have proved useful, limitations still exist.

On page 806 of this issue, Fire and colleagues[6] describe a remarkable and surprising technique for inhibiting gene function in *C. elegans*. They turned off a specific gene in progeny worms by microinjecting double-stranded RNA (dsRNA) complementary to the coding region of the gene into the gonads of adult animals. Using a well-characterized gene, *unc-22*, which encodes a non-essential myofilament protein, they showed that injection of dsRNA produced a phenotype characteristic of *unc-22* inhibition — twitching.

In a series of well-controlled studies, the authors also found that injection of dsRNA targeted to a reporter gene for green fluorescent protein resulted in a dramatic — and specific — decrease in protein production. Furthermore, when they injected dsRNA targeted to another gene, *mex-3*, the result was a loss of *mex-3* RNA in early-stage embryos. In other words, at the levels of phenotype, RNA and protein, the interference with gene expression was specific and reproducible.

Perhaps most astounding is the phenomenon that the dsRNA causes gene inhibition. Previously[3], Fire and co-workers had been puzzled by the fact that antisense RNA alone — which is often used to inactivate sense messenger RNA — was only marginally effective. Furthermore, results using the antisense RNA were mimicked by injection of sense RNA, a control in their studies. They later found out that these data could be largely explained by an artefact of the transcription process that was used to generate the antisense and sense RNAs; namely, dsRNA fragments.

Additional experiments by Fire *et al.*, designed to shed light on the possible mechanism of the dsRNA-mediated inhibition, painted an even more mystifying picture. For example, even when only a few copies of the dsRNAs are present in each cell, they are active against highly abundant RNAs. This indicates that the interference occurs either by a catalytic mechanism or at the chromosomal level — and not by a conventional antisense mechanism. The authors also found that only dsRNAs that are complementary to coding regions of the gene are active, and not, for example, those targeted to introns or promoter regions. This argues against a generalized mechanism involving chromosomal inactivation, such as chromosomal deletion. Moreover, dsRNA interference seems to cross cellular boundaries with

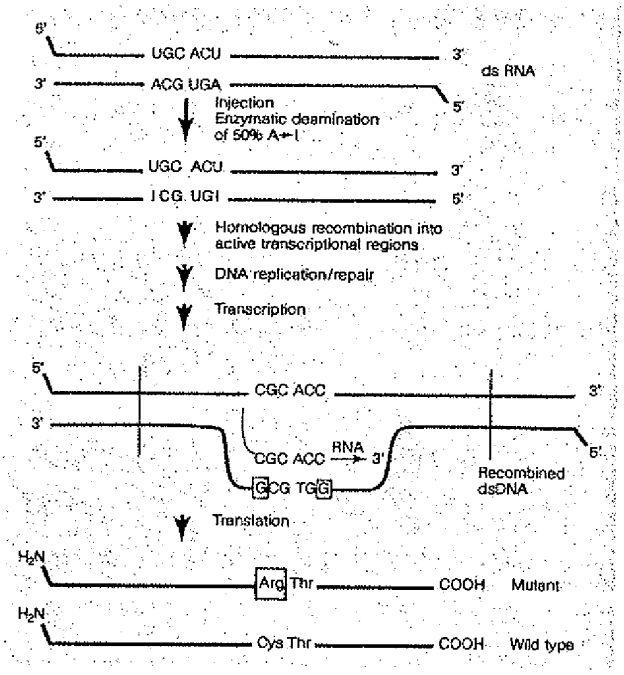

Figure 1 Possible mechanism for inhibition of gene expression in *C. elegans* by double-stranded RNA. Fire *et al.*[6] have convincingly shown that, at the phenotype, RNA and protein levels, dsRNA-mediated interference with gene expression is specific and reproducible. Perhaps, on injection into worms, dsRNA is modified by dsRNA adenosine deaminase. Transfer of this information back into the chromosome may occur by a recombination event. After replication and mismatch repair, transcription and translation result in mutant proteins that have impaired function.

ease. Gene inhibition was observed in progeny when dsRNA was injected into the body cavity of the adult (gonadal injections had been thought to be necessary), and in somatic tissues of young adults after injection into their body cavity.

What kind of mechanism have Fire and colleagues uncovered? This is not the first puzzle posed by dsRNA. Almost ten years ago, Bass and Weintraub[7] and Wagner *et al.*[8] discovered an enzyme that binds dsRNA and deaminates adenosines in the duplex to inosines. After a feverish hunt for the cellular function of the dsRNA adenosine deaminase, it was found to be involved in the post-transcriptional editing of messages. Inosines are read by the cellular machinery as guanosine, so the enzyme could alter the genetic make-up of mRNA (reviewed in refs 9, 10).

Could this dsRNA adenosine deaminase be involved in a complicated pathway that results in gene inhibition in *C. elegans*? Quite possibly. The enzymatic activity has been found in *C. elegans*, and would probably treat the injected dsRNA as a substrate. A specialized homologous recombination system would be needed, which would use the modified dsRNA to transfer the genetic alterations into the chromosome (Fig. 1).

This model fits some of the data: modification of adenosines to inosines alters the genetic make-up of the injected dsRNA; transfer of this information into the genome by recombination would affect coding (but not intronic) regions; and mutations introduced by the inosine substitutions would affect the ability to detect mRNA and, at least partially, the function of the protein. These mutations could account for the surprising result that only a few copies of dsRNA are required per cell, because they would have an effect at the level of the chromosome. Of course, such a model is a stretch of the imagination and is not supported by all of the data. For example, attempts to use homologous recombination with dsDNA in *C. elegans* have largely failed[3].

Fire and colleagues[6] have uncovered a complex and intriguing mode of regulation in *C. elegans*. Does dsRNA perform a biological function in *C. elegans* (and is this function titrated out by the microinjected dsRNA)? Does a similar phenomenon exist in other organisms? What would happen if transgenic animals or plants were generated expressing both the sense and antisense strands of a transgene? A similar mode of action would not be suspected to occur in mammals, because injection of dsRNA is often used as a control for antisense experiments, albeit at the individual cell (and not organism) level. Nevertheless, perhaps specific 'knockouts' can be generated this way, for organisms in which genetic material cannot be delivered by microinjection. Whatever the mechanism might be, dsRNA-mediated inhibition of gene expression will provide a useful alternative for working out gene function in *C. elegans* and, maybe, in other animals and plants. □

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Liquid crystals

New designs in cholesteric colour

Peter Palffy-Muhoray

Since their discovery in 1888, cholesteric liquid crystals have been subject to considerable attention, resulting in applications in ink and paint technologies, flat-panel displays and thermal imaging. Writing in *Advanced Materials*[1], Tamaoki and co-workers describe a new technique for rewritable full-colour image recording on thin cholesteric films. The low-molecular-weight compound they have developed for this purpose is a cholesteric glass, which is stable at room temperature and which could have applications in optics as well as information display and storage.

The optical properties of cholesterics have made them useful in display[2,3] and laser technologies[4] as well as in the visual arts[2]. In reflected light, cholesterics show intense iridescent colours with a metallic sheen, as seen on scarab beetles. In these materials, rod-like molecules are orientated, on the average, parallel to one another in a given plane, so that the direction of orientation varies linearly with position in the direction normal to the plane. This results in a spatially periodic twisted helical structure as shown in

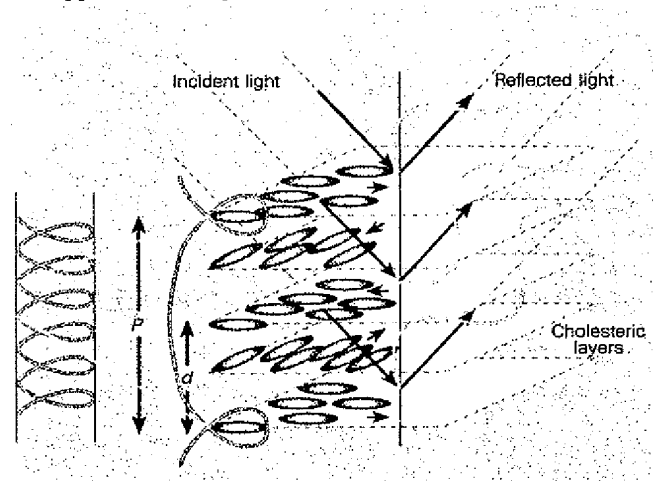

Figure 1 Sketch of cholesteric structure, showing the dependence of molecular orientation on position. The tip of a vector indicating local molecular orientation traces out a helix. Reflected light waves satisfying the Bragg condition emerge in-phase and add constructively. In the work discussed here, Tamaoki *et al.*[1] have developed a cholesteric glass that is rewritable and stable at room temperature (see Fig. 3).

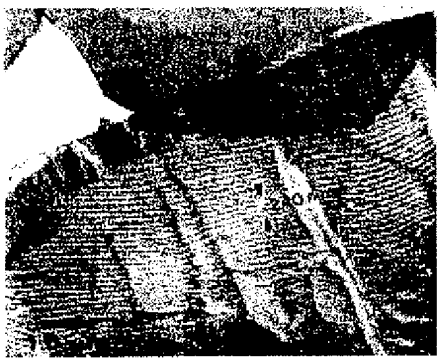

Figure 2 Transmission electron micrograph of freeze-fractured helical cholesteric. The pitch is 240 nm. (From ref. 12.)

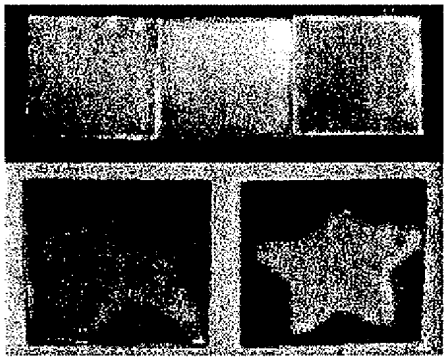

Figure 3 Photographs of thermally addressed and quenched cholesteric solid films. (From ref. 1.)